PATENT

S/N 09/096,749

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Shohei Koide	Examiner:	Larry R. Helms, Ph.D.
Serial No.:	09/096,749	Group Art Unit:	1642
Filed:	June 12, 1997	Docket:	109.034US1
Title:	ARTIFICIAL ANTIBODY POLYPEPTIDES		

Declaration of Shohei Koide under 37 C.F.R. § 1.132

Commissioner for Patents
Washington, D.C. 20231

I, Shohei Koide, Ph.D, declare and say as follows:

1. I am the inventor of the above-referenced U.S. Patent Application.

2. In addition to the examples presented in the original filing, I have performed additional experiments that further support the pending claims. I prepared mutant FNfn10 proteins that contain glycine insertions or glycine-rich insertions in the AB, BC, CD, DE, EF or FG loops. Glycines and the glycine-rich sequence (Gly-Gly-Met-Gly-Gly) were chosen to be inserted because glycine insertions are generally highly destabilizing. Glycines have a high degree of conformational freedom (large entropy) in the unrestrained state. It is thus energetically unfavorable to restrain glycines by folding of a protein, because of a high entropic penalty. Therefore these insertion mutations are expected to provide useful guidelines as to whether these loops can be extensively modified for the engineering of binding proteins.

3. Mutant proteins were prepared using the Kunkel mutagenesis or polymerase chain reaction (PCR) metagenesis methods, and the conformational stability was determined using guanidine hydrochloride-induced denaturation, as described in (Koide, A., Bailey, C. W., Huang, X. & Koide, S. (1998) The fibronectin type III domain as a scaffold for novel binding proteins. *J. Mol. Biol. 284*, 1141-1151). Figure 1 attached to the present Declaration shows effects of these mutations on the conformational stability of FNfn10. Specifically, Figure 1 depicts denaturation curves of FNfn10 and its variants containing additional glycine residues in one or more loops.

Denaturation reactions were monitored using tryptophan fluorescence and analyzed according to the two-state model, as described in Koide *et al.* (1998). Experiments were performed in 10 mM sodium citrate buffer pH 6.0 containing 100 mM sodium chloride at 30 °C (the AB, BC, DE and FG insertions) or in 20 mM sodium phosphate buffer pH 7.0 containing 100 mM sodium chloride (the CD and EF loop insertions). The conformational stability of these molecules do not significantly change between the two set of measurement conditions.

4. The following Table A summarizes the free energy of unfolding in the absence of guanidine hydrochloride.

Table A: Effects of glycine insertions on the conformational stability of FNfn10

Protein	ΔG (kcal/mol)
Wild Type	7.70±0.09
Four glycines in the FG loop	6.91±0.15
Eight glycines in the FG loop	6.62±0.11
Four glycines in the DE loop	5.66±0.21
Four glycines in the BC loop and eight glycines in the FG loop	6.09±0.17
Four glycines in the AB loop	8.23±0.09
Gly-Gly-Met-Gly-Gly insertion in the CD loop	7.87±0.18
Gly-Gly-Met-Gly-Gly insertion in the EF loop	6.32±0.10

These results show that some of the insertions decrease the stability of FNfn10, but these mutant proteins still retain sufficient degrees of conformational stability to remain folded. For example, even the least stable protein (the DE loop mutant) is more than 99.99% folded in the buffer solution. Thus, these results demonstrate that it is feasible to make mutant proteins in which the loops of the FNfn10 protein contain deletions, insertions and replacements.

5. **Selection of AB-loop monobodies that bind to the human estrogen receptor α**

Additional monobodies were created that have variable AB-loop regions. The following protocol was used:

Materials: 17β-estradiol (E2) was purchased from Sigma. An anti-LexA antibody was kindly provided by Dr. E. Golemis (Fox Chase Cancer Center). Secondary antibodies were purchased from Pierce. The ERα cDNA clone was kindly provided by the late Dr. A. Notides (University of Rochester Medical Center).

Strains and media: Yeast strains EGY48, *MATα his3 trp1 ura3 leu2::6LexAop-LEU2*, and RFY206, *MATa his3Δ200 leu2-3 lys2Δ201 trp1Δ::hisG ura3-52*, have been described (Gyuris *et al.*, *Cell 75*, 791-803 (1993); Finley & Brent, *Proc. Natl. Acad. Sci. USA 91*, 12980-12984 (1994)) and were purchased from OriGene Technologies (Rockville, MD). Yeast was grown in YPD media or YC dropout media following instructions from Origene Technologies (The DupLEX-A™ Yeast Two-Hybrid System Manual). Manipulation of *Escherichia coli* was according to Sambrook *et al.* (Sambrook *et al.*, Molecular Cloning: A laboratory manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor (1989)).

Construction of yeast two-hybrid vectors and a monobody library: The method of Brent and others in vector construction was essentially followed (Mendelsohn & Brent, *Curr. Opin. Biotechnol. 5*, 482-486 (1994); Golemis & Serebriiskii, Two-hybrid system/interaction trap in *Cells: A laboratory manual* Ed.) pp69.1-40, CSH Laboratory Press, Cold Spring Harbor, NY (1997); Colas & Brent, *Trends Biotechnol. 16*, 355-363 (1998)). The synthetic gene for FNfn10 (Koide *et al.*, *J. Mol. Biol. 284*, 1141-1151 (1998)) was subcloned in the plasmid pYESTrp2 (Invitrogen, Carlsbad, CA) so that FNfn10 is fused C-terminal to the B42 activation domain (pYT45). The plasmid encoding a LexA-fusion protein of residues 297-595 of ERα was constructed by subcloning the ERα gene fragment in the plasmid pEG202 (Origene Technologies) using standard PCR cloning methods (pEGERα297-595). Although ERα itself has a weak transcriptional activation function in yeast (Chen *et al.*, *Biochem. Pharmacol. 53*, 1161-1172 (1997)), these constructs did not activate the *LEU2* reporter gene to an extent that confers *LEU*$^+$ phenotype in the yeast EGY48.

A monobody library was constructed by inserting seven diversified residues between Pro15 and Thr16 in the AB loop (residue numbering according to Koide *et al.*, *J. Mol. Biol. 284*, 1141-1151 (1998)). The DNA segment corresponding to these seven residues of FNfn10 in the plasmid pYT45 was randomized using the NNS codon (N denotes a mixture of A, T, G, C, and S denotes a mixture of G and C) by Kunkel mutagenesis (Kunkel *et al.*, *Methods Enzymol. 154*,

367-382 (1987)). The yeast strain EGY48 was transformed with this plasmid to produce a library containing approximately $2x10^6$ independent clones.

Library screening: The yeast strain RFY206 harboring pEGERα297-595 and a *LacZ* reporter plasmid, pSH18-34 (Origene Technologies), was mated with EGY48 containing the monobody library as described previously (Finley & Brent, *Proc. Natl. Acad. Sci. USA 91*, 12980-12984 (1994)). Diploid cells that contain an ERα-binding monobody were selected using the *LEU*$^+$ phenotype on minimal dropout media (Gal Raf –leu –his –ura –trp). The library screening was performed in the presence of 1 μM E2. Colonies grown after three days of incubation at 30 °C were further tested for galactose-dependence of the *LEU*$^+$ phenotype and β-galactosidase activity. The plasmids coding for a monobody were recovered from yeast clones following instructions supplied by Origene Technologies, and the amino acid sequences of monobodies were deduced by DNA sequencing.

β-Galactosidase assay: Quantitative assays were performed as follows. The yeast strain RFY206 was first transformed with pEGERα297-595 and pSH18-34 and subsequently with a derivative of the pYT45 plasmid encoding a particular monobody. Yeast cells were grown overnight at 30°C in the YC Glc –his –ura –trp media. The culture was then spun down, the media were discarded, and the cells were resuspend in YC Gal Raf –his –ura –trp media containing a ligand at a final cell density of 0.2 OD_{660nm} in a total volume of 175 μl in the wells of a deep 96-well plate. The ligand (E2) concentration was 1 μM. After incubating for six hours at 30°C with shaking, 175 μl of β-galactosidase assay buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, 0.27% β-mercaptoethanol, 0.004% SDS, 4mg/ml 2-nitrophenyl-β-D-galactosidase, 50% Y-PER (Pierce Chemical Co., Rockford, IL)) was added to the culture, incubated at 30°C, then the reaction was stopped by adding 150 μl of 1M Na_2CO_3. After centrifugation, OD_{420} was measured and the β-galactosidase activity was calculated. *See*, attached Figure 2.

6. The results of the protocol in ¶ 5 above were the following. Two clones that bind to the E and F domain of the human estrogen receptor α (ERα) were obtained from the screening. Their amino acid sequences are shown below.

	amino acid sequence in the AB loop
	P_{15}-------T_{16}
wild type	PXXXXXXXT
library	WTWVLRE
clone A1	WVLITRS
clone B1	

In this table, "–" denotes a gap in the wild-type sequence where an additional residue was inserted in the library, and "X" denotes a diversified position in the library. Quantitative β-galactosidase assays were performed. It has been shown that β-galactosidase activity is well correlated with the strength of the interaction in the yeast two-hybrid system (Estojak *et al.*, *Mol. Cell. Biol.* 15:5820-5829 (1995)). The two selected monobodies bind to ERα in the presence of E2 but not in the absence of E2, indicating a high degree of binding specificity of these monobodies. In contrast, the wild-type Fn3 showed no detectable binding to ERα in the absence or presence of E2.

7. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

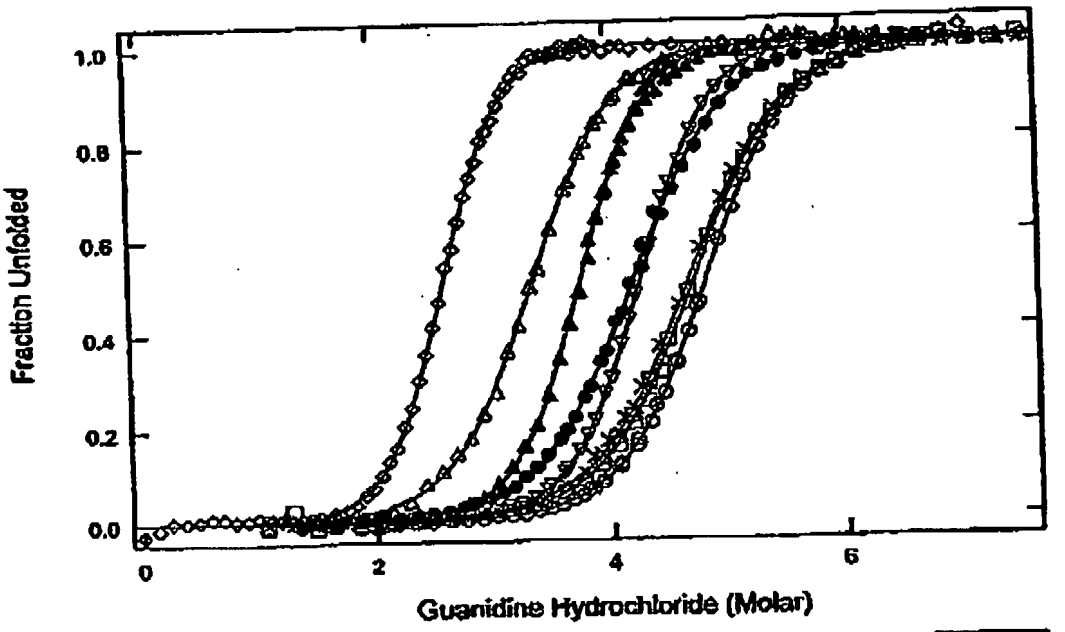
Date October 23, 2001 By Shohei Koide
Shohei Koide, Ph.D.

CERTIFICATE UNDER 37 CFR 1.8: The undersigned hereby certifies that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail, in an envelope addressed to: Commissioner of Patents, Washington, D.C. 20231, on October __, 2001.

Name

Signature

Figure 1



Guanidine hydrochloride-induced unfolding of FNfn10 mutants. The fraction of unfolded molecule is plotted as a function of guanidine hydrochloride concentration.

Figure 2